Dissociation and Reassembly of the Vacuolar H+-ATPase Complex from Oat Roots[1]

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ABSTRACT

Conditions for the dissociation and reassembly of the multisubunit vacuolar proton-translocating ATPase (H+-ATPase) from oat roots (*Avena sativa* var Lang) were investigated. The peripheral sector of the vacuolar H+-ATPase is dissociated from the membrane integral sector by chaotropic anions. Membranes treated with 0.5 molar KI lost 90% of membrane-bound ATP hydrolytic activity; however, in the presence of Mg^{2+} and ATP, only 0.1 molar KI was required for complete inactivation of ATPase and H+-pumping activities. A high-affinity binding site for MgATP (dissociation constant = 34 micromolar) was involved in this destabilization. The relative loss of ATPase activity induced by KI, KNO_3, or KCl was accompanied by a corresponding increase in the peripheral subunits in the supernatant, including the nucleotide-binding polypeptides of 70 and 60 kilodaltons. The order of effectiveness of the various ions in reducing ATPase activity was: KSCN > KI > KNO_3 > KBr > K-acetate > K_2SO_4 > KCl. The specificity of nucleotides (ATP > GTP > ITP) in dissociating the ATPase is consistent with the participation of a catalytic site in destabilizing the enzyme complex. Following KI-induced dissociation of the H+-ATPase, the removal of KI and MgATP by dialysis resulted in restoration of activity. During dialysis for 24 hours, ATP hydrolysis activity increased to about 50% of the control. Hydrolysis of ATP was coupled to H+ pumping as seen from the recovery of H+ transport following 6 hours of dialysis. Loss of the 70 and 60 kilodalton subunits from the supernatant as probed by monoclonal antibodies further confirmed that the H+-ATPase complex had reassembled during dialysis. These data demonstrate that removal of KI and MgATP resulted in reassociation of the peripheral sector with the membrane integral sector of the vacuolar H+-ATPase to form a functional H+ pump. The ability to dissociate and reassociate *in vitro* may have implications for the regulation, biosynthesis, and assembly of the vacuolar H+-ATPase *in vivo*.

An H+-ATPase[2] is located in the vacuolar membrane (tonoplast) of higher plant cells (24). This H+-ATPase generates an electrochemical gradient of protons across the vacuolar membrane (acidic and positive inside) that drives the accumulation of a variety of solutes via secondary-active and passive transporters (23). The plant vacuolar H+-ATPase is a member of the vacuolar class of ATPases (V type) that acidify endomembrane compartments of eucaryotic cells (for a recent review see ref. 6).

Enzymes of this class are large, multimeric, and contain a peripheral and membrane integral sector (6, 23). Vacuolar H+-ATPases from red beet (16) and mung bean (14) consist of 7 to 10 subunits. The oat root H+-ATPase has a M_r of 650,000 and is composed of 10 subunits (26). Six subunits from the oat enzyme were identified as peripheral because they could be removed from the membrane by chaotropic reagents such as KI. Dissociation of the H+-ATPase results in a loss of ATP hydrolytic activity and solubilizes a high mol wt complex (M_r = 540,000) of five peripheral subunits (70, 60, 44, 36, and 29 kD). Another peripheral subunit (42 kD) is also solubilized by KI, but it is not associated with this peripheral complex following dissociation (26). The membrane integral sector of the vacuolar ATPase consists of mainly the N,N'-dicyclohexylcarbodiimide-binding 16 kD proteolipid (10, 12, 21).

Although chaotropic anions, such as KI, were previously shown to inactivate and dissociate the plant vacuolar H+-ATPase (12, 22), recent studies indicated that MgATP or ATP interacts with chaotropic anions in dissociating vacuolar ATPases from bovine chromaffin granules (15), bovine coated vesicles (1), *Neurospora* (3), and red beet (16) vacuolar membranes. Here we report that the binding of MgATP to a high-affinity site on the oat H+-ATPase enhanced the dissociation of the enzyme by chaotropic reagents. Furthermore, the dissociated ATPase could be reassembled by removing the KI and MgATP using dialysis. The reassembled H+-ATPase was active in both H+-transport and ATP hydrolysis. To our knowledge, this represents the first report of reassembly of a plant vacuolar H+-ATPase *in vitro*.

MATERIALS AND METHODS

Plant Material

Oat seeds (*Avena sativa* L. var Lang) were germinated in the dark over an aerated solution of 0.5 mM $CaSO_4$. Roots were harvested after 4 d of growth.

[2] Abbreviations: H+-ATPase, proton-translocating adenosine triphosphatase; BTP, bis-tris propane or 1,3-bis[tris(hydroxymethyl)methylamino]propane.

Preparation of Vacuolar Membrane Vesicles

Membrane vesicles were prepared using differential and density gradient centrifugation according to the procedure of Randall and Sze (19) as modified by Ward and Sze (26). Oat roots were homogenized in 50 mM Hepes-BTP, pH 7.4, 250 mM sorbitol, 6 mM EGTA, 0.2% BSA, 1 mM DTT, and 0.1 mM PMSF at a medium to tissue ratio of 2 mL/g and strained through cheesecloth. After removing whole cells and mitochondria (13,000g pellet), microsomal membranes were collected by centrifugation at 60,000g (Beckman[3]/SW 28, r_{max}). Microsomal membranes were resuspended in resuspension buffer (25 mM Hepes-BTP, pH 7.2, 250 mM sorbitol, 1 mM DTT, 0.1 mM PMSF) and layered over 6% Dextran (mol wt 80,000) in resuspension buffer. After centrifugation at 70,000g (Beckman SW 28.1, r_{max}), vacuolar membranes were collected at the Dextran interface. Three to 4 mg of vesicle protein was typically obtained from 90 g of oat roots. Vacuolar membranes were diluted 10-fold into resuspension buffer, collected by centrifugation at 85,000g (SW 28, r_{max}), and suspended in resuspension buffer at 6 to 8 mg protein/mL. All procedures were conducted at 4°C. Protein was estimated by the Bradford method (5) using BSA as a standard.

Dissociation Treatments

The effects of pretreatment of tonoplast vesicles with Mg^{2+}, nucleotides, and K^+ salts (KI, KNO_3, or KCl) were investigated using two procedures. In most experiments, membrane vesicles (approximately 0.25 mg protein) were diluted into 2 mL of resuspension buffer with additions as indicated in the legends. After mixing, the vesicles were incubated at 4°C for 1 h and collected by centrifugation at 185,000g (TY65, r_{max}) for 1 h. The pellets were suspended in 100 μL of resuspension buffer. Usually, 20 μL was used for measuring ATPase activity and 10 μL (one-tenth of the total fraction) was used for SDS-PAGE or immunoblot analysis. One-tenth of the supernatant fraction (200 μL) was analyzed by SDS-PAGE or immunoblot.

For the time-course experiments, vesicles were pretreated in resuspension buffer alone or with KI and MgATP. The mixture (33 μL) was then diluted 30-fold into ATPase assay medium at the times indicated.

Reassembly Experiments

Membrane vesicles (approximately 0.4 mg protein) were incubated in the presence and absence of 0.15 M KI, 5 mM $MgSO_4$, and 5 mM ATP-BTP in 2 mL of resuspension buffer at 4°C. After 1 h, glycerol (100%) was added to a final concentration of 10% (v/v). Samples were dialyzed against 300 volumes of dialysis buffer (20 mM Hepes-BTP, pH 7.0, 10% glycerol, 2 mM 2-mercaptoethanol, 0.2 mM EGTA, 1 mM EDTA, 0.1 mM PMSF) at 4°C using Spectra/Por CE (Spectrum) with a mol wt cutoff of 15,000. At various times,

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[3] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply approval to the exclusion of other products that may also be suitable.

samples of 50 or 200 μL were taken for ATP hydrolysis or H^+-transport assays, respectively.

ATPase Activity

The release of ADP by ATPase was measured spectrophotometrically (19). The assay mixture contained 30 mM Hepes-BTP, pH 7.0, 50 mM KCl, 3 mM ATP-BTP, 4.5 mM $MgSO_4$, 0.66 mM phospho*enol*pyruvate, 5 units/mL pyruvate kinase, 12 units/mL lactate dehydrogenase, 0.2 mM NADH, 90 μM ammonium molybdate, 0.2 mM NaN_3, 0.1 mM Na_3VO_4, 10 μg gramicidin D/mL, and 40 to 60 μg vesicle protein. Absorbance was monitored at 340 nm for 4 min at 25°C. Assays were done in the absence or presence of 50 mM NO_3^--BTP, pH 7.0, and the results are presented as NO_3^--sensitive ATPase activity (25).

H$^+$ Pumping Activity

Proton transport into tonoplast vesicles was measured by the quenching of acridine orange fluorescence. Reaction mixtures contained 15 mM Hepes-BTP, pH 7.0, 190 mM sorbitol, 0.33 mM EGTA-BTP, 50 mM KCl, 5 mM $MgSO_4$, 1.5 mM ATP-BTP, and 0.5 μM acridine orange in a final volume of 1 mL. ATP-driven H^+ pumping was initiated by the addition of $MgSO_4$. Fluorescence (495 nm excitation and 525 nm emission) was monitored at 22°C using a Farand System III spectrofluorometer.

Gel Electrophoresis and Immunostaining

All samples were precipitated with 10% TCA, washed with 100% acetone, and boiled for 4 min in sample buffer containing 62 mM Tris-Cl, pH 6.8, 10% (v/v) glycerol, 8 M urea, 5% 2-mercaptoethanol, and 0.002% (w/v) bromphenol blue. Laemmli gels (12% acrylamide), 15 × 20 cm, were run at 13 mA for 1 h then 18 mA for 4 h.

After electrophoresis, gels were soaked in 25 mM Tris, 194 mM glycine, 15% methanol, pH 8.2, for 20 min followed by blotting to Immobilon-P (Millipore) at 40 V, 140 mA, overnight at 4°C using a Bio-Rad blotting apparatus. Following incubation for 1 h in PBS containing 1 M glycine, 5% dry milk, and 1% BSA, the membrane was washed three times in TPBS (PBS with 0.05% Tween-20) and incubated for 1 h with monoclonal antibodies (diluted with TPBS containing 1% dry milk as indicated in figure legends). The membrane was then washed as before, probed with goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase, and developed with 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium (20).

Monoclonal Antibody Production and Screening

Three female BALB/c mice were injected intraperitoneally with 15 to 20 μg of oat vacuolar H^+-ATPase purified as previously described (26) and emulsified with Freund's complete adjuvant. Three booster injections of 15 μg purified protein in complete adjuvant were given at 2- to 3-week intervals. One of the three mice produced antibodies to the vacuolar ATPase as determined by western blots probed with serum from tail bleeds. A final injection without adjuvant

was given to that mouse, and 4 d later the spleen was removed. The spleen cells were fused with myeloma cells (P3x63aG8.653) as previously described (9) and seeded into 20 96-well culture plates. Briefly, spleen cells were mixed with myeloma cells (taken from a log growth phase) at a 5:1 ratio and copelleted in a 50 mL conical centrifuge tube. This process was repeated and the medium was aspirated completely. Fusion was carried out in a 50-mL conical tube in a water bath at 37°C by adding dropwise 1.5 mL of prewarmed 45% PEG (mol wt 4000) in RPMI 1640 medium over a period of 45 s. RPMI 1640 medium was supplemented with 5.7% each of Nu-serum (Collaborative Research, Inc., Bedford, MA), CPSR-3 controlled processed serum replacement (Sigma), and Hyclone fetal serum, defined (Hyclone Laboratory, Inc., Logan, UT). Hybridomas were selected by growth in hypoxanthine-aminopterin-thymidine medium.

Hybridoma supernatants were screened for recognition of tonoplast proteins or purified oat H⁺-ATPase by western blotting. Tonoplast vesicle protein (150 μg) was electrophoresed on 12% acrylamide preparative gels (Mini Protean II, Bio-Rad). Gels were blotted to Immobilon-P (Millipore) overnight at 40 V and blocked as described above. The membranes were then clamped into a multichannel apparatus (Miniblotter 28, Immunetics, Cambridge, MA). Fifty microliters of monoclonal supernatants diluted 1:2 with TPBS containing 1% milk were placed in each channel and incubated for 1 h. The membranes were washed, probed with alkaline phosphatase-labeled goat anti-mouse immunoglobulin G antibodies, and developed as described above. Positive hybridomas were screened again against purified vacuolar H⁺-ATPase (10 μg/ preparative gel) by the same method. Clones that tested positive were expanded and retested.

Characterization of Monoclonal Antibodies

To determine if the antibodies reacted with the native ATPase, Immobilon P membrane was incubated with vacuolar membrane protein (10 μg/cm²) for 15 min at 4°C, and then blocked (5% milk, 1% BSA, 1 M glycine in PBS). The membrane was then clamped into a slot-blot apparatus (Schleicher & Schuell) and culture supernatants (1:2 dilution) were added to the wells. After a 1-h incubation, the membrane was washed and developed as described above. To determine if the antibodies inhibited ATPase activity, tonoplast vesicles were incubated with an equal volume of hybridoma culture supernatants for 20 min at 4°C. Samples (100 μL) were assayed for NO₃⁻-sensitive ATPase activity.

### RESULTS

Dissociation of the Vacuolar H⁺-ATPase Complex

Treatment of plant vacuolar membranes with KI separates the peripheral and integral sectors of the vacuolar H⁺-ATPase and inactivates the ATP hydrolysis and H⁺-transport activities of the enzyme (12, 22, 26). To understand the interaction between the peripheral complex and the integral components of the H⁺-ATPase, we have examined the conditions required for the dissociation of peripheral subunits.

KI-Induced Inactivation Is Temperature Dependent

We first tested the effect of incubation time and temperature on the loss of NO₃⁻-sensitive ATPase activity. In the absence of KI and MgATP, there was little loss in ATPase activity from vesicles preincubated at either 4 or 20°C (Fig. 1). However, membranes incubated with KI at 4°C rapidly lost ATP hydrolytic activity, showing a 50% drop in activity within 15 min (Fig. 1). At 20°C, the enzyme activity decreased at a slower rate, requiring 30 min for 50% inhibition. These results are similar to the cold inactivation observed for the H⁺-ATPases from bovine chromaffin granules (15) and red beet vacuole membrane (16), and suggest that cold sensitivity may be a common feature of eukaryotic vacuolar H⁺-ATPases. Because about 80 to 90% of the total activity was lost after 1 h at 4°C (Fig. 1), these conditions were used for further analyses. To prevent interference of chaotropic ions with the ATPase assay, the membranes were pelleted, suspended in resuspension buffer, and tested for NO₃⁻-sensitive ATPase activity.

MgATP Enhanced the Dissociation of the Vacuolar H⁺-ATPase by Chaotropic Anions

Loss of membrane-bound ATPase activity was greatest after treatment with KI, Mg²⁺, and ATP together. Membranes treated with 0.1 M KI alone or with 5 mM MgSO₄ or 5 mM ATP lost only 30% of the ATPase activity, whereas 89% of
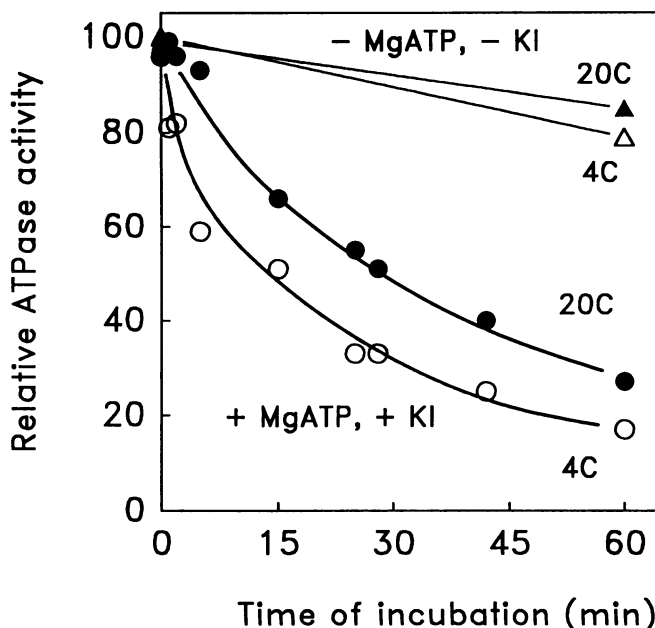
Figure 1. KI-induced inactivation of the vacuolar H⁺-ATPase was temperature dependent. Tonoplast vesicles were incubated in resuspension buffer alone (△, ▲) or buffer containing 5 mM MgSO₄, 5 mM ATP-BTP, with 0.1 M KI (○, ●) at either 4 or 20°C. At the times indicated, NO₃⁻-sensitive ATPase activity was measured at 25°C after diluting 33 μL vesicles 30-fold into the assay mixture. At time zero, 3.3 mM KI in the reaction mixture had no effect on ATPase activity. Points are means of two experiments and 100% activity = 2.7 nmol/min.

the H$^+$-ATPase activity was lost when vacuolar membrane vesicles were incubated with 0.1 M KI, 5 mM MgSO$_4$, and 5 mM ATP (Table I). This suggested that MgATP binding to the H$^+$-ATPase destabilized the enzyme and enhanced the chaotropic effect of KI.

The decrease of membrane-bound ATPase activity, enhanced by MgATP, was accompanied by an increase in dissociation of the peripheral subunits. Figure 2 demonstrates that both Mg^{2+} and ATP were required to enhance the solubilization of peripheral H$^+$-ATPase subunits. Six polypeptides (70, 60, 44, 42, 36, and 29 kD) previously identified as peripheral subunits of the H$^+$-ATPase (26) were solubilized (Fig. 2).

Besides I$^-$, several other anions were effective in decreasing vacuolar ATPase activity in the presence of MgATP. At 0.1 M, anions decreased ATPase activity with the following order of effectiveness: SCN$^-$ > I$^-$ > NO$_3^-$ > Br$^-$ > CH$_3$COO$^-$ > SO$_4^{2-}$ > Cl$^-$ (Table II). This order follows the Hofmeister series (8, 17, 22) and suggested that the chaotropic potency of the anions determined the degree of inhibition. It should be noted that chaotropic ion-induced loss of ATPase activity by NO$_3^-$ is distinct from the rapidly reversible pseudo-competitive inhibition observed at low concentrations of NO$_3^-$ (22). Direct NO$_3^-$ inhibition of ATP hydrolysis is detected in less than 1 min and only 8 mM NO$_3^-$ is needed to achieve 50% inhibition (19, 25).

Just like KI, the concentration of KNO$_3$ or KCl required to inhibit the enzyme was significantly lowered in the presence of MgATP (Fig. 3). ATPase activity was decreased 50% by 0.2 M and 0.04 M KI in the absence and presence of MgATP, respectively. Maximum (90%) inhibition occurred at 0.5 M KI in the absence of MgATP and 0.1 M KI in the presence of MgATP. With MgATP, NO$_3^-$ and Cl$^-$ caused 50% inhibition at concentrations of 0.02 and 0.15 M, respectively.

To probe for ATPase subunits released from vacuolar membranes, we utilized monoclonal antibodies recently produced against the oat vacuolar H$^+$-ATPase. Monoclonal antibodies, 7D2 and 2E7, reacted specifically with 70 and 60 kD poly-
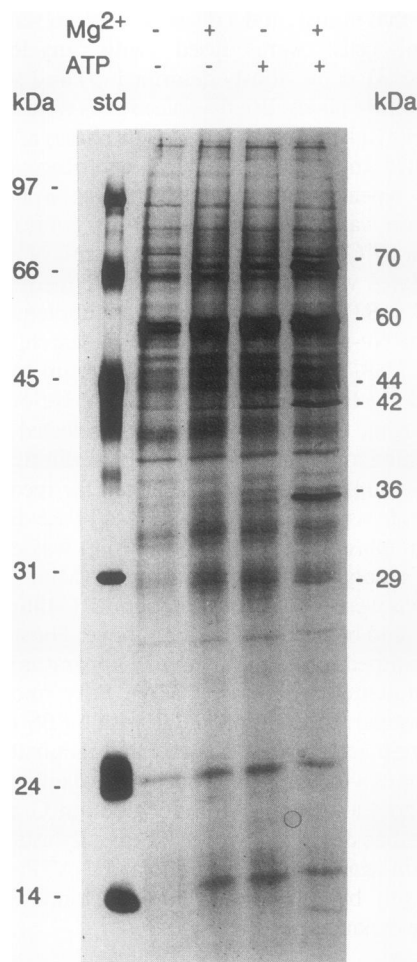
Figure 2. Mg^{2+} and ATP enhanced the solubilization of peripheral H$^+$-ATPase subunits by KI. Vesicles were pretreated with 0.1 M KI alone; with 5 mM Mg^{2+}, 5 mM ATP; or both. Membranes were then pelleted. The supernatants were analyzed by SDS-PAGE and the gel was silver stained. Peripheral subunits of the H$^+$-ATPase (26) are indicated in the right margin.

Table I. *KI-Induced Loss of Membrane-Bound Vacuolar ATPase Activity Was Enhanced with Mg^{2+} and ATP*

Vacuolar membranes (approximately 0.25 mg protein) were incubated in 2 mL of resuspension buffer with the following additions at 4°C. KI was added at 0.1 M and MgSO$_4$ and ATP at 5 mM, final concentration. After 1 h, the vesicles were pelleted, suspended in 100 μL resuspension buffer, and 20 μL was assayed immediately for ATPase activity. Means are from three to four experiments and 100% activity = 4.0 nmol/min.

Treatment	NO$_3^-$-Sensitive ATPase Activity
	% ± *SE*
None	100
KI	77 ± 4.2
MgSO$_4$ + ATP	71 ± 4.4
KI + MgSO$_4$	70 ± 10.4
KI + ATP	67 ± 2.6
KI + MgSO$_4$ + ATP	11 ± 1.5

peptides, respectively, of both vacuolar membrane vesicles and of the partially purified H$^+$-ATPase from oat (26) (Fig. 4). Antibody 7D2 (anti-70 kD) reacted with the native H$^+$-ATPase as well as with the denatured subunit on western blots, and also inhibited 62% of the NO$_3^-$-sensitive ATP hydrolysis activity of vacuolar membrane vesicles. In contrast, antibody 2E7 recognized the SDS-denatured 60 kD subunit on western blots but not the native enzyme and, consistent with this, did not inhibit ATPase activity. In addition, 2E7 recognized a single polypeptide of about 60 kD on western blots of microsomal protein from various plant tissues, including *Arabidopsis* shoots, corn roots, red beet storage tissue, and soybean roots, whereas 7D2 reacted only with oat (data not shown).

The relative amount of the 70 and 60 kD subunits released by KI with or without MgATP corresponded well to the relative loss of ATPase activity (Fig. 5). These results support the notion that the dissociation of the ATPase complex by

Table II. *Effect of Chaotropic Anions with MgATP on the Loss of Membrane-Bound Vacuolar ATPase Activity*

Vacuolar membranes (approximately 0.25 mg protein) were incubated in 2 mL of resuspension buffer containing 5 mM $MgSO_4$, 5 mM ATP-BTP, and different K^+-salts (0.1 M) for 1 h at 4°C. Vesicles were pelleted and assayed for NO_3^--sensitive ATPase activity. Relative effectiveness was estimated as the percentage of activity lost relative to KI (100%).

Treatment (0.1 M)	NO_3^--Sensitive ATPase Activity	Relative Effectiveness
	% ± SE	%
None	100	0
KCl	62 ± 13.4	13
K_2SO_4	59 ± 3.8	18
K-Acetate	53 ± 1.7	28
KBr	40 ± 5.5	50
KNO_3	18 ± 2.1	87
KI	11 ± 1.5	100
KSCN	8 ± 1.0	103

chaotropic ions was enhanced by MgATP and that the release of peripheral subunits resulted in loss of membrane-bound ATPase activity. The immunostaining method used in this study is quantitative over only a limited range of antigen amount (7). However, detectable amounts of 60 and 70 kD subunits in the supernatants resulted from treatments that caused greater than 40% inactivation. Thus, the appearance of 70 and 60 kD subunits in the supernatants (Fig. 5) was related to loss of hydrolysis activity in the pellets (Fig. 3). Under conditions that completely inactivated ATP hydrolysis, detectable amounts of both 60 and 70 kD subunits remained in the pellets. This is most probably due to the presence of inside-out vesicles in the membrane preparations used in this study (12, 22).

Nucleotide Specificity and High-Affinity MgATP Binding Site

In the presence of 0.1 M KI, MgATP decreased ATPase activity with a dissociation constant of about 34 μM (Fig. 6). In these experiments, $MgSO_4$ was maintained at 2 mM above the ATP concentration to ensure that all of the ATP was present as MgATP. The same dissociation constant was obtained with equimolar Mg^{2+} and ATP (data not shown). The affinity of this MgATP-binding site is about sevenfold higher than the K_m for catalysis (19), indicating that catalytic turnover may not be required for MgATP to promote dissociation.

Membranes pretreated with 5 mM GTP or ITP, in the presence of 0.1 M KI and 5 mM $MgSO_4$, also lost ATPase activity. The order of effectiveness of the nucleotides tested in three to four experiments was:

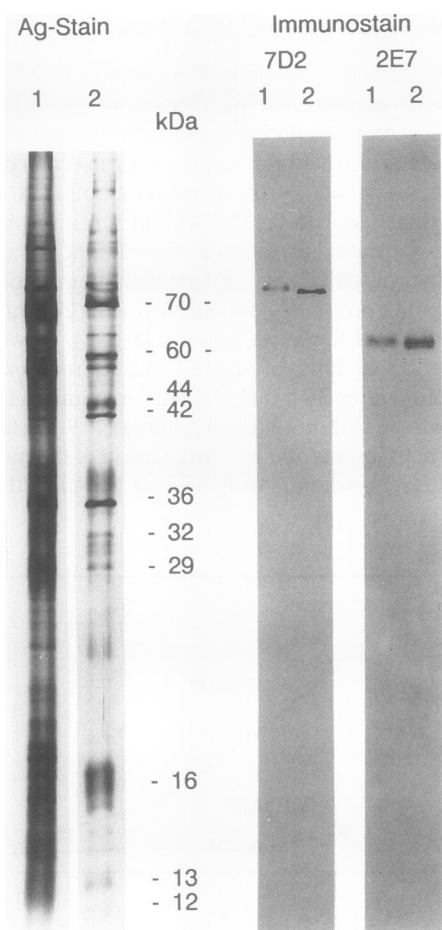ATP[100] > GTP[59] > ITP[44]

> ADP[5] = AMP[0] = none[0].

This order is similar to the substrate specificity of the membrane-bound and partially purified vacuolar H⁺-ATPase from oats: ATP[100] > GTP[40] > ADP[12] = AMP[12] (19, 25) and suggested that a catalytic site was involved in destabilization of the H⁺-ATPase.

Reassembly of the Vacuolar H⁺-ATPase

Because chaotropic anions in the presence of MgATP induced the dissociation and inactivation of the ATPase, we wondered if activity could be recovered by removal of these reagents as demonstrated for bovine clathrin- coated vesicles (18). To test this possibility, the membrane-bound H⁺-ATPase was first dissociated with 0.15 M KI and 5 mM MgATP, and the mixture was then dialyzed to remove KI, Mg^{2+}, and ATP. The ATP hydrolysis activity of vacuolar membranes preincubated in buffer only (−KI) was stable during 24 h of dialysis, losing only 15% of the initial activity (Fig. 7). However, the ATP hydrolysis activity of the KI-treated membranes increased twofold after 24 h dialysis. Half-maximal recovery of activity occurred within 3 h during dialysis. We tested if this recovery of activity was due to reorientation of the membrane vesicles. After dissociation with KI and MgATP, the mem-
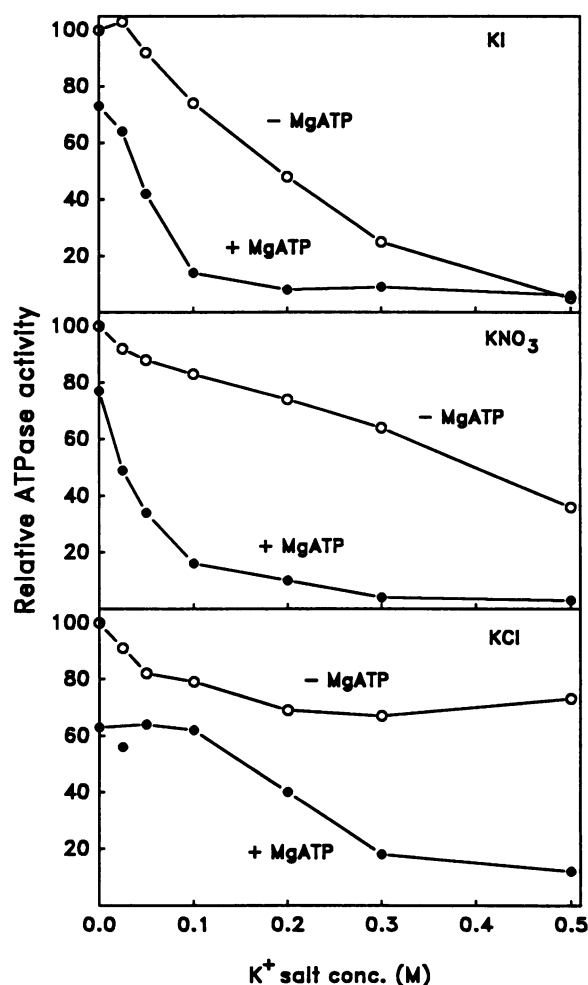
Figure 3. MgATP lowered the concentration of chaotropic anions required to decrease ATPase activity. Tonoplast vesicles (approximately 0.25 mg protein) were incubated at 4°C with various concentrations of KI, KNO_3, or KCl, in the presence or absence of 5 mM $MgSO_4$ and 5 mM ATP. The vesicles were pelleted and assayed for NO_3^--sensitive ATPase activity. Points are means of two to three experiments and 100% activity = 4.4 nmol/min.
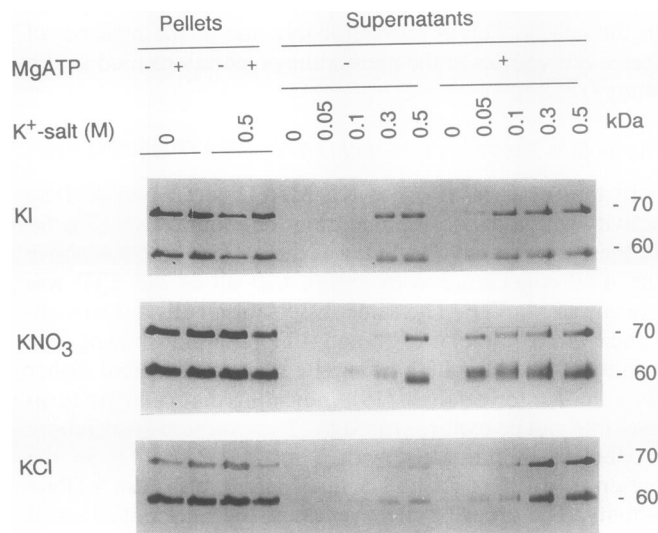
activity decreased only 15% in buffer-treated vesicles (Fig. 7), the decrease in pH gradient formation would indicate that the vesicles became leaky to H^+. In contrast, the initial rate of fluorescence quenching by the H^+-translocating pyrophosphatase was not decreased by KI as shown before (12, 22, 26). After 6 h dialysis, PPi-driven H^+-translocation also decreased markedly (by 40%) for the vesicles preincubated in buffer but not for KI-treated vesicles (data not shown). These results support the idea that during dialysis the control membranes became more leaky to protons than KI-treated membranes. The reason for this is not clear; perhaps KI inhibited lipase or protease activities. However, the recovery of ATP-dependent H^+ pumping activity in KI-treated vesicles demonstrates that the peripheral sector had reassociated with the integral sector.

To confirm the reassembly of the vacuolar ATPase after removal of KI, we tested for a coincident removal of peripheral subunits from the soluble fraction during dialysis. Vacuolar membrane vesicles were treated with KI and MgATP to dissociate the H^+-ATPase, and then collected by centrifugation before or after 6 h of dialysis. The presence of 70 and 60 kD subunits in the supernatants were monitored by western blotting. Negligible amounts of these subunits were detected by silver stain or immunostain in supernatants from buffer-treated membranes (Fig. 9). As shown above (Fig. 5), membranes treated with KI and MgATP released six subunits into solution, including the 70 and 60 kD subunits detected by immunostain (Fig. 9). Following 6 h dialysis, the levels of these peripheral subunits in the supernatants decreased dramatically. Due to the presence of inside-out vesicles in the

Figure 4. Monoclonal antibodies reacted specifically with the 70 and 60 kD subunits of oat vacuolar H^+-ATPase. Tonoplast vesicles (6 μg protein, lane 1) and partially purified H^+-ATPase (0.4 μg, lane 2) were separated by SDS-PAGE. Triton-solubilized H^+-ATPase was partially purified by gel filtration followed by step gradient elution from Q-Sepharose FF. Gels (12% acrylamide) were silver stained or blotted to Immobilon P. Western blots were probed with monoclonal antibodies 7D2 (1:100 dilution) or 2E7 (1:200).

branes were pelleted, resuspended, and dialyzed separately from the supernatants. ATPase activity was not recovered in either the pellet or supernatant (Fig. 7). Because recovery of membrane-bound ATPase activity required both the soluble and membrane fractions following dissociation, these results suggested that the vacuolar H^+-ATPase complex had reassembled.

If the peripheral sector was functionally reassociated with the membrane sector of the vacuolar ATPase, we would expect to see recovery of proton pumping activity following dialysis. KI-treated vesicles lost their ability to generate a pH gradient (acid inside) as shown by acridine orange fluorescence quenching (Fig. 8, refs. 22, 26). However, after 6 h dialysis, the initial rate of fluorescence quenching (relative units/min) increased from 2.0 to 5.5, consistent with the increase in ATP hydrolysis activity (Fig. 7). Interestingly, proton pumping in control (buffer treated) vesicles decreased from 18 to 10 after 6 h dialysis (Fig. 8). Because ATPase

Figure 5. Loss of membrane-bound ATPase activity induced by chaotropic anions was coincident with the solubilization of H^+-ATPase subunits. Vacuolar membrane vesicles (0.25 mg protein) were treated with KI, KNO_3, or KCl in the presence or absence of 5 mм MgATP. After centrifugation, equal volume fractions (10%) of the pellets and supernatants were analyzed by SDS-PAGE (12% polyacrylamide) and blotted to Immobilon PVDF. The 70 and 60 kD subunits of the H^+-ATPase were identified by immunostaining with a mixture of antibodies 7D2 (1:100) and 2E7 (1:200).
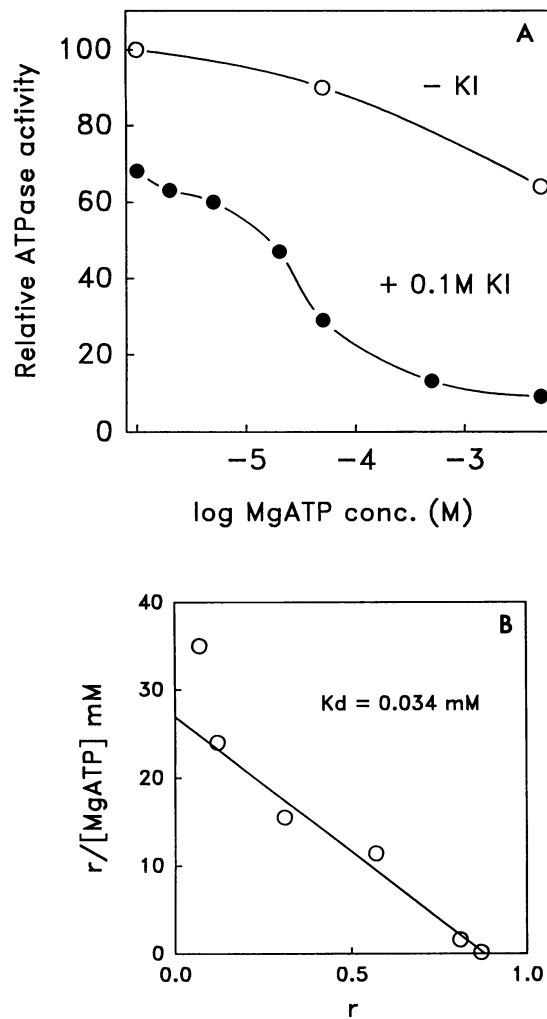
Figure 6. Binding of MgATP to a high-affinity site on the H⁺-ATPase destabilized the enzyme complex. Tonoplast vesicles (0.25 mg protein) were incubated for 1 h at 4°C in resuspension buffer with or without 0.1 M KI at various MgATP concentrations. MgSO₄ concentration was 2 mM higher than that of ATP. After centrifugation, the pellets were assayed for NO₃⁻-sensitive ATPase activity. Points are means from three experiments and 100% activity = 3.8 nmol/min. A, Relative ATPase activity; B, Scatchard analysis where r is the fraction of activity remaining after KI treatment.

preparations, an increase in membrane-bound peripheral subunits following dialysis was difficult to detect (see Fig. 5). However, these data indicated that reassociation of the peripheral subunits with the membrane led to recovery of both ATP hydrolysis (Fig. 7) and H⁺-transport activities (Fig. 8). Therefore, removal of KI and MgATP facilitated the binding of the peripheral sector with the integral sector of the vacuolar ATPase to produce a fully functional H⁺-pumping ATPase.

DISCUSSION

The oat vacuolar H⁺-pumping ATPase could be inactivated by dissociating the peripheral complex from the integral components and then fully reactivated by reassembling the two

sectors. The optimal conditions required for the selective release of peripheral subunits was a combination of KI, Mg²⁺, and ATP (Table I, Figs. 2 and 5), whereas removal of these reagents promoted reattachment (Figs. 7–9). We recently demonstrated that the H⁺-ATPase from oat has a molecular mass of 650 kD with a large peripheral sector of about 540 kD. This sector consists of six polypeptides of 70, 60, 44, 42, 36, and 29 kD, which are solubilized from the membrane with KI (26). The membrane sector is made up of multiple (six) copies of the N,N'-dicyclohexylcarbodiimide-binding 16 kD proteolipid plus a few minor polypeptides (e.g. 13 and 12 kD). The 16 kD subunit remains membrane bound after KI treatment (12, 22) and is solubilized only by chloroform/methanol (10, 21) or detergents.

The presence of MgATP caused the H⁺-ATPase to be more susceptible to dissociation by chaotropic anions. However, the order of effectiveness of anions in dissociating the H⁺-ATPase was similar to the Hofmeister series either in the absence (22) or the presence of MgATP (Table II). This is consistent with the suggestion that nucleotide-induced conformational changes destabilize the vacuolar H⁺-ATPases from Neurospora and bovine-coated vesicles (1, 3). Interestingly, ATP alone is sufficient to destabilize the bovine-coated vesicle and Neurospora vacuolar H⁺-ATPases (1, 3), whereas
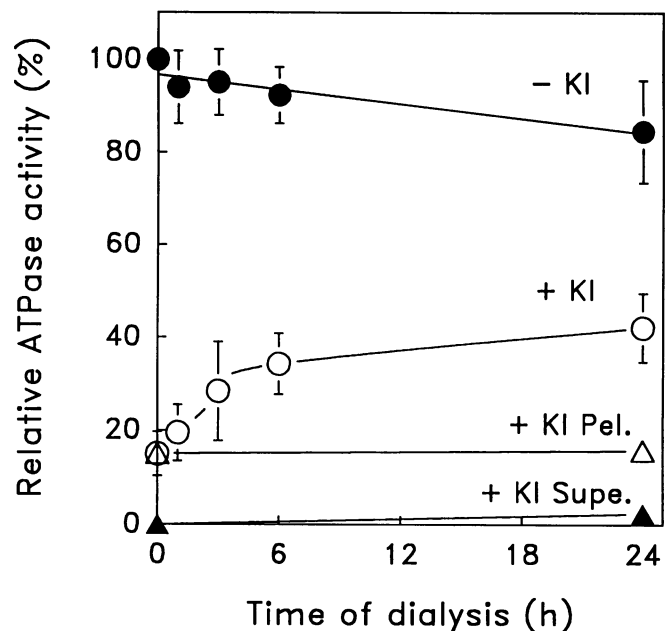
Figure 7. Removal of KI and MgATP from the dissociated enzyme restored ATPase activity. Vacuolar membrane vesicles (0.3–0.6 mg) were incubated at 4°C for 1 h in the presence (+ KI) or absence (−KI) of 0.15 M KI and 5 mM MgSO₄ in a final volume of 1 mL. The total mixture was then dialyzed for varying periods, and ATPase activity was measured. Another sample treated with KI and MgATP as above was separated by centrifugation, and the pellet was suspended in 1 mL buffer. The pellets (+ KI Pel.) and supernatants (+ KI Supe.) were dialyzed independently for varying periods and NO₃⁻-sensitive ATPase activity was measured. Points are means (± SE) of three to four experiments (100% activity = 5.2 nmol/min). Pel. and Supe. are means of two experiments.
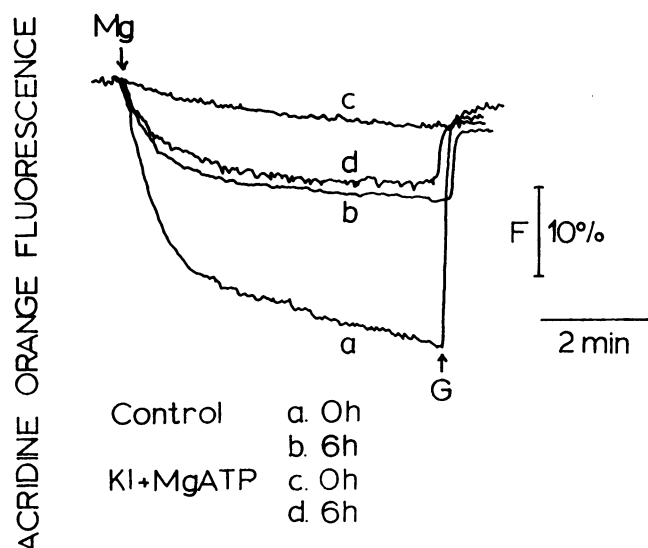
Figure 8. Proton-pumping activity of the dissociated vacuolar H^+-ATPase was restored following removal of KI. Membrane-bound H^+-ATPase was incubated for 1 h either in resuspension buffer alone (Control) or in KI and MgATP as in Figure 7. Samples were assayed for H^+ transport before dialysis (a, c) or after 6 h of dialysis (b, d). Transport was initiated by 5 mM Mg (5 μL of 1 M MgSO$_4$). pH gradient was collapsed by G (5 μL of 1 mg/mL gramicidin D).

the dissociation of the enzyme from oat, red beet (16), and bovine chromaffin granule (15) required both Mg^{2+} and ATP. The reason for this difference is unclear, but may reflect structural differences in the ATP-binding site involved in this effect.

The site of nucleotide-binding, which is functional in destabilizing the H^+-ATPase, remains unclear. For the oat vacuolar H^+-ATPase, a dissociation constant of 34 μM was estimated for the MgATP binding site involved in enhanced dissociation (Fig 6, A and B). As the K_m for catalysis of the oat H^+-ATPase was 250 μM (19, 25), distinct sites might be involved in catalysis and destabilization. However, several lines of evidence suggest that MgATP binding to a high-affinity catalytic site increases the susceptibility of the H^+-ATPase to dissociation. Two sites responsible for ATP hydrolysis were identified for the bovine-coated vesicle H^+-ATPase with K_m values of 83 and 790 μM (1). Also, ^{18}O-exchange measurements of the vacuolar H^+-ATPases of yeast and *Neurospora* support a model of multiple catalytic sites with decreasing affinity and a reaction mechanism similar to F_1F_o-ATPases (11). As both GTP and ITP are substrates for vacuolar ATPases (2, 19), the order of effectiveness of nucleotides ATP > GTP > ITP in dissociating the oat vacuolar ATPase would be consistent with the participation of a catalytic site in the destabilization of the enzyme. Finally, 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole blocks dissociation of the *Neurospora* vacuolar H^+-ATPase (3) at concentrations that specifically label the 70 kD catalytic subunit of vacuolar H^+-ATPases from oat, corn, and *Neurospora* (4, 20). However, vacuolar H^+-ATPases may have ATP-binding sites other than the catalytic site, as the 60 kD subunit of the red beet vacuolar H^+-ATPase can bind to the ATP analog, 3-*O*-(4-benzoyl)benzoyladenosine 5'-triphosphate

(13). Thus, although our results are consistent with the involvement of a high-affinity catalytic site in destabilization of the H^+-ATPase, other nucleotide-binding sites cannot be ruled out at this time.

The time-course for recovery of the oat ATP hydrolysis activity (Fig. 7) is similar to that found for the reassembly of the bovine-coated vesicle H^+-ATPase (18). In that study, 80% of H^+-transport activity was recovered after 24 h of dialysis to remove KI and MgATP. As reported here, the initial rate of H^+-transport more than doubled following dialysis. Compared with vesicles pretreated with buffer only, the recovery of H^+-transport activity was between 30 and 55% (based on control H^+-transport activity before and after dialysis, respectively).

Dissociation of both the oat vacuolar H^+-ATPase and the bovine-coated vesicle H^+-ATPase results in the solubilization of a large complex with five peripheral subunits and an additional subunit of 41 to 42 kD, which is not associated with this complex (18, 26). During reassembly of the bovine-
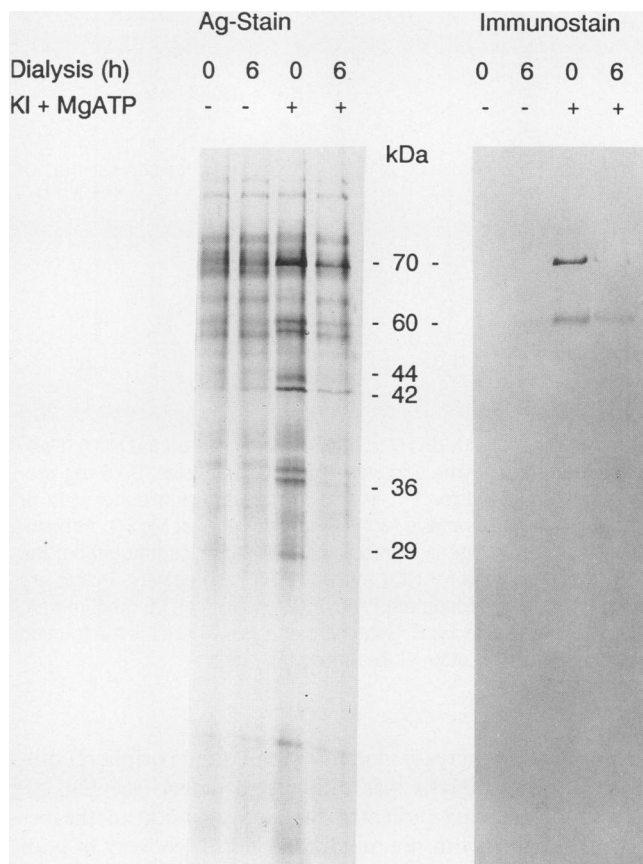
Figure 9. Solubilized H^+-ATPase subunits disappeared from supernatants after removal of KI. Vacuolar membrane vesicles (0.75 mg) were preincubated with KI and MgATP (as in Fig. 7) in a final volume of 1.5 mL. Before and after 6 h of dialysis, 0.5 mL aliquots were taken and the membranes separated by centrifugation (180,000g, Beckman TLA 100.3) for 20 min. One-tenth of the supernatants were separated by SDS-PAGE and gels were either silver stained or blotted to Immobilon P and probed with a mixture of monoclonal antibodies 7D2 (1:100) and 2E7 (1:200).

coated vesicle H$^+$-ATPase, both the peripheral complex and the 41 kD subunit are reassociated with the membrane integral sector to form a functional enzyme (18). Similarly, all six peripheral subunits of the oat H$^+$-ATPase are removed from solution by dialysis (Fig. 9). This suggests that three separate components of the vacuolar H$^+$-ATPase have reassembled: (a) the membrane integral sector, (b) the peripheral complex, and (c) the 42 kD peripheral subunit.

The ion-induced dissociation and reassembly of the vacuolar ATPase complex seen *in vitro* may have biological and physiological implications. Dissociation may be responsible in part for the chilling-induced inactivation of the vacuolar H$^+$-ATPase *in vivo*. Because the high-affinity MgATP-binding site involved in destabilization of the H$^+$-ATPase would be occupied under most physiological conditions, changes in ionic strength or ionic composition (*e.g.* NO$_3^-$ leakage from the vacuole) during chilling injury (27) might lead to dissociation of the H$^+$-ATPase. During exposure at 2°C, the vacuolar ATPase activity and H$^+$-pumping activity of mung bean suspension culture cells decreased in 10 h to 20 and 0% of control, respectively. This inactivation was fully reversible within 1 h after the cells were returned to 26°C and did not require *de novo* protein synthesis (27). These results are consistent with a model in which chilling-induced dissociation of the vacuolar H$^+$-ATPase is followed by reassembly once the cells are restored to 26°C. This ability to dissociate and reassociate may reflect a mechanism used in the regulation, biosynthesis, and assembly of the vacuolar-type H$^+$-ATPase *in vivo*.

ACKNOWLEDGMENTS

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